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Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers

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Stem cell transplantation is used widely in the management of a range of diseases of the hemopoietic system. Patients are immunosuppressed profoundly in the early posttransplant period, and reactivation of cytomegalovirus (CMV) remains a significant cause of morbidity and mortality. Adoptive transfer of donor-derived CMV-specific CD8⁺ T cell clones has been shown to reduce the rate of viral reactivation; however, the complexity of this approach severely limits its clinical application. We have purified CMV-specific CD8⁺ T cells from the blood of stem cell transplant donors using staining with HLA-peptide tetramers followed by selection with magnetic beads. CMV-specific CD8⁺ cells were infused directly into nine patients within 4 h of selection. Median cell dosage was 8.6×10^3 /kg with a purity of 98% of all T cells. CMV-specific CD8⁺ T cells became detectable in all patients within 10 d of infusion, and TCR clonotype analysis showed persistence of infused cells in two patients studied. CMV viremia was reduced in every case and eight patients cleared the infection, including one patient who had a prolonged history of CMV infection that was refractory to antiviral therapy. This novel approach to adoptive transfer has considerable potential for antigen-specific T cell therapy.

CMV reactivation is a significant clinical problem after allogeneic stem cell transplantation (SCT; reference 1). Antiviral drugs can reduce the incidence of early-onset CMV disease, but are associated with substantial toxicity and the development of late-onset CMV disease. CMV reactivation arises because of impaired CMV-specific immunity and CD8⁺ CTLs are believed to play the predominant role in suppressing viral replication. This has led to the development of clinical protocols whereby CMV-specific CD8⁺ T cell clones are cultured from the transplant donor and transferred to the patient after transplantation (2–5). This has proven effective in the prevention of reactivation and treatment of CMV infection that is unresponsive to antiviral therapy. However, this procedure has not

been adopted widely because of the significant technical and financial demands of extensive ex vivo T cell culture.

CMV-specific CD8⁺ T cells are found at high frequency in the blood of healthy CMV-seropositive individuals and typically represent 0.5–4% of the CD8⁺ T cell pool (6). Magnetic beads allow the selection of antigen-specific T cells using HLA-peptide tetramers (7); this offers the prospect of selecting CMV-specific CD8⁺ T cells directly from the blood of transplant donors and transferring them into the patients without ex vivo manipulation. Here, we have selected CMV-specific T cells from nine SCT donors and infused these directly into transplant recipients. Adoptive transfer was followed by considerable expansion of CMV-specific CTLs in vivo and subsequent control of viremia.

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RESULTS AND DISCUSSION

CMV-specific CTLs can be purified by HLA-peptide tetramers and remain functional

CMV-specific CTLs were selected from CMV-seropositive donors by staining PBMCs with HLA-peptide tetramers containing peptides from CMV pp65 or IE-1, followed by selection using magnetic beads. 10 healthy CMV-seropositive laboratory donors were recruited to evaluate the efficiency of large-scale selection. 0.41–12.3% (median 2.5%) of CD8⁺ T cells stained with tetramer before selection. This increased to 97.8–99.9% (median 98.8%) after positive selec-

tion (Fig. 1 A), and represented a median of 94% of the total live cell population. The sensitivity of selection was good; an average of 61% of CMV-specific CTLs was recovered. The functional activity and proliferative potential of positively selected CTLs was confirmed in vitro (Fig. 1, B–D).

CMV-specific CTLs may be administered directly to patients after selection

Nine patients were treated with adoptive therapy; six patients had received the transplant from an HLA-matched sibling and three patients had received stem cells from an

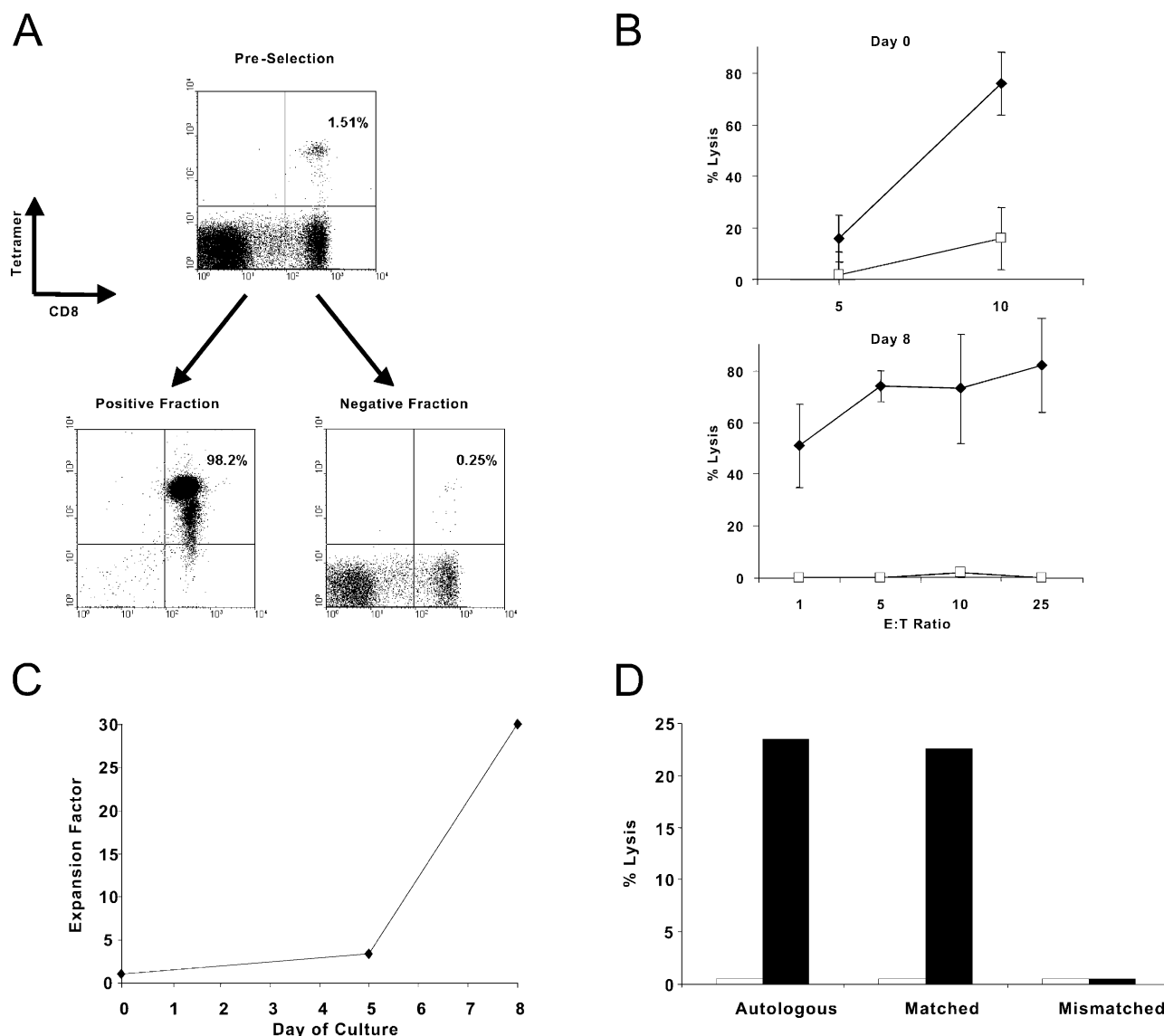


Figure 1. Selection of CMV-specific CTLs from blood samples using large-scale magnetic separation. (A): HLA-peptide tetramer staining of donor PBMCs before selection and in the positive and negative fractions after selection. (B) Cytotoxicity assay of positively selected cells performed directly after selection or after 8 d of culture on autologous peptide-loaded cells. ♦, 0.5 μM peptide; □, DMSO control. (C) Growth characteristics of cells in the positive fraction reveal a 30-fold expansion after 8 d. Cells

were expanded with autologous lymphoblastoid cells and allogeneic feeder cells. (D) Positively selected CMV-specific CTLs lyse autologous and HLA-matched fibroblasts, which have been infected with CMV. CTLs were cultured for 8 d and subsequently were tested on fibroblasts that were infected with CMV. The E:T ratio in all cases was 2:1. □, Mock-infected fibroblasts; ■, CMV-infected fibroblasts. All results are representative of at least three independent experiments.

Table I. Virology, cell selection, treatment, and immune recovery data

Patient	Clinical details				Selection data			Outcome			
	Primary diagnosis	Transplant protocol	Day cells infused	ALC at infusion	Specificity infused	Total cells infused/kg	Purity	Day after infusion of CTL peak	Peak CTL	Maximum grade of GvHD	Antiviral administration
$\times 10^9/L$				$\% CD3^+ cells$			$tetramer^+ cells/\mu l$				
1	WM	BC	40	0.1	NLV	3.1×10^3	98.5	12	8.4	0	yes
2	AML	FMC	18	0.3	YSE	1.47×10^4	92.6	18	156	0	no
3	CLL	FMC	23	1.1	YSE	3.1×10^3	95.6	19	42	0	no
4	CML	Cy, TBI	34	0.8	YSE + ELR	1.23×10^3	99.5	16	15	II	yes
5	HD	FMC	29	0.4	TPR + NLV	1.67×10^3	88.0	27	44	I	no
6	CML	Cy, Bu	55	0.6	NLV	8.6×10^3	98.0	4	101	0	no
7 ^a	CML	Cy, TBI	246	1.2	NLV	3.3×10^4	99.2	6	2	0	pretherapy
8 ^a	ALL	Cy, TBI	247	0.55	IPS	1.22×10^4	10 ^b	26	1.1	0	pretherapy
9	ALL	FMC	21	0.2	ELR	1.0×10^4	95.4	114	105	II	pretherapy

^aPatient 7 had been refractory to ganciclovir and patient 8 had been refractory to cidofovir.

^bPurity was reduced due to the low frequency of the CMV-specific CD8⁺ T cells in the donor (0.08% of CD8⁺ cells). The depletion of cells not binding tetramer was >3 log. CMV epitopes are as follows: ELR-IE1₁₉₉₋₂₀₇; IPS-pp65₁₂₃₋₁₃₁; NLV – pp65₄₉₅₋₅₀₃; TPR-pp65₄₁₇₋₄₂₆; YSE-pp65₃₆₃₋₃₇₃.

ALC, absolute lymphocyte count; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BC, BEAM/Campath-1H; Bu, busulfan; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; Cy, cyclophosphamide; FMC, fludarabine melphalan Campath-1H; HD, Hodgkin's disease; TBI, total body irradiation; WM, Waldenström's macroglobulinemia.

HLA-unrelated donor (Table I). The first six patients and patient 9 received adoptive transfer of CTLs after the initial episode of viral reactivation, whereas patients 7 and 8 were treated for persistent viremia. CMV-specific CTLs were selected from 250 ml of peripheral blood or a leukapheresis product from the stem cell donor. Positively selected cells were infused into patients within 4 h of selection. $1.2 \times 10^3/kg$ to $3.3 \times 10^4/kg$ of selected CTLs were administered with a median T cell purity of 95.6% (Table I). No toxicity was observed after cell infusion.

CMV-specific immune reconstitution was observed after infusion of CMV-specific CTLs

Immune reconstitution of CMV-specific CTLs was monitored by staining blood samples with HLA-peptide tetramer before infusion and after adoptive transfer. Examples of viral load and immune recovery in the first three patients are shown in Fig. 2; complete data are presented in Table I. CMV-specific CTLs were detected in all patients after adoptive transfer and were maintained during the follow-up period of up to 24 mo. The peak CTL response was seen between 12 and 30 d after adoptive transfer with a range of 1–156 cells/ μl of blood. Persistence of infused cells was determined using the TCR CDR3 sequence of the infused cells as a clonotypic marker. Analysis was possible only in patients 2 and 7 because of limited availability of patient and donor samples. No clonotype-specific T cells were detectable in either patient before adoptive transfer but they were present at every time point after immunotherapy (Fig. 2 B; see Fig. 3 C). Patient 2 demonstrated persistence of CTLs to at least 100 d after infusion, and the intensity of the PCR product increased over time in both patients, which suggested expansion of the infused cells.

The functional activity of CMV-specific CTLs was measured by the use of intracellular staining for IFN- γ after peptide stimulation. IFN- γ -producing cells were seen at an early time point after adoptive transfer, although the proportion of tetramer-binding cells that expressed IFN- γ gradually increased in the first 6 mo after transfer (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20040613/DC1>).

Reduction in CMV viremia was seen in all patients

CMV viral load was monitored weekly after the first episode of reactivation. In the first seven patients and patient 9, CMV reactivation resolved completely after adoptive transfer of CTL. Patient 8 documented marked reduction in CMV viral load in association with CMV-specific immune reconstitution. No case of CMV disease was seen.

Of the six patients who received stem cells from a sibling donor, four did not require antiviral therapy; however, in accordance with the trial protocol, two patients (33%) were treated with ganciclovir (GCV) after adoptive transfer, one of whom had received corticosteroids within 1 wk of therapy. In comparison, GCV was required in 9 of 11 patients (81.8%) in a control group who did not receive adoptive transfer during the same period. The difference in the proportion of patients who required GCV was 48%; the 95% confidence interval for this value is 4–93%. This confidence interval is wide and reflects the small number of patients in the trial; however, the fact that it remains above zero indicates that it is highly likely that treatment does reduce the proportion of patients who require GCV treatment.

CMV-specific immune reconstitution is known to be impaired markedly in patients who receive stem cells from an unrelated donor (8); the three patients with unrelated donors in our study were particularly informative in demonstrating the therapeutic potential of adoptively transferred

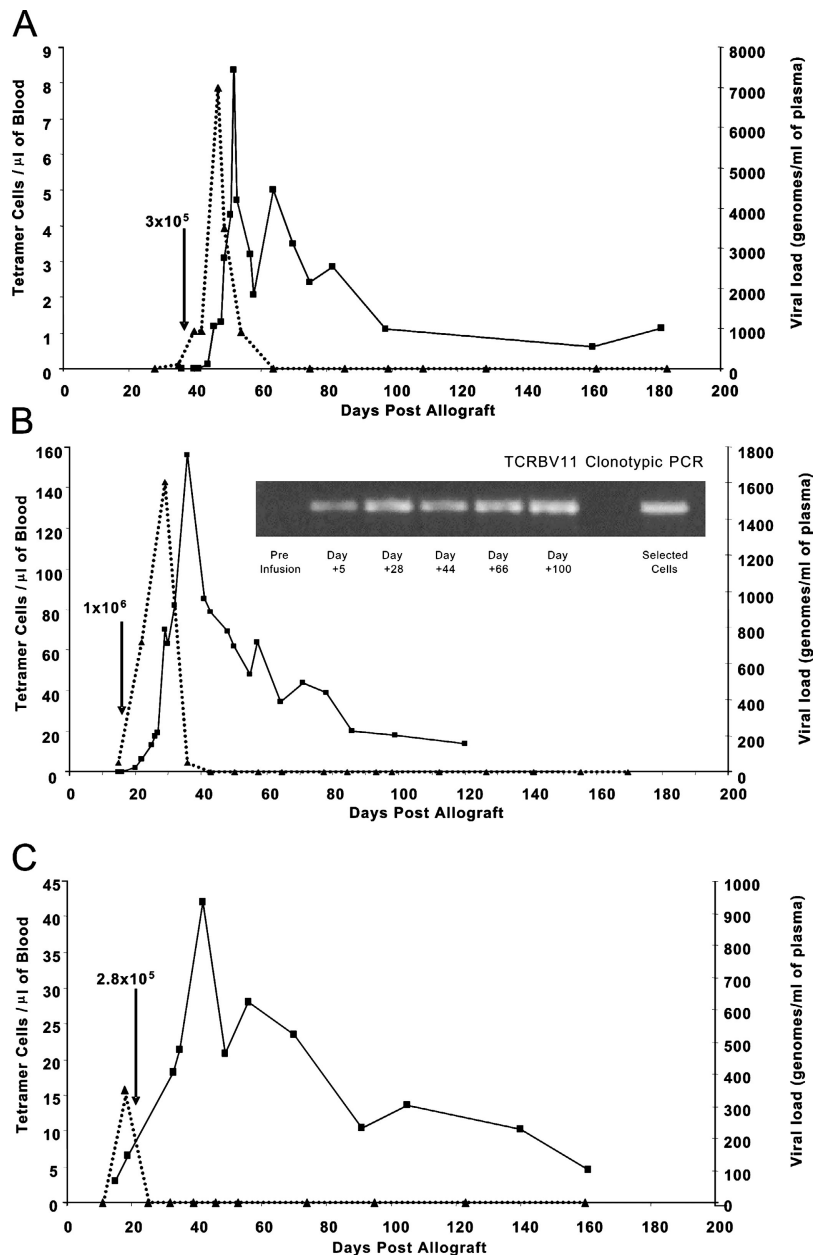


Figure 2. CMV-specific immune reconstitution and CMV viremia after adoptive transfer of CMV-specific CTLs. The vertical bar indicates the timing of adoptive transfer and the absolute number of CMV-specific CTLs administered. Data from patients 1, 2 and 3 (A–C) are shown. ■, Absolute count of tetramer⁺ T cells; ▲, viral load. Persistence of the infused T cells was assessed in patient 2 by PCR detection of the clonotypic TCR CDR3 sequence of the infused cells in PBMC samples that were taken from the patient. FACS analysis of donor PBMCs demonstrated that 80% of the CMV-

specific CTLs expressed the TCRBV11 chain. *TCRBV11*-specific PCR was performed on positively selected donor CMV-specific CTLs, and the PCR products were cloned and sequenced to determine the dominant TCR clonotype. A single TCR CDR3 sequence, YLCARFDGRAGETQYFGPG, comprised 90% of all *TCRBV11* sequences. A clonotype-specific primer (5'-gtttgacggagggc-cggtga-3') was synthesized and used with a *TCRBC* primer for clonotype-specific PCR which was performed on patient PBMCs that were isolated before infusion and at days 5, 28, 44, 66, and 100 after infusion.

CTLs (Fig. 3). Patient 7 had developed primary CMV infection and experienced 4 mo of persistent CMV reactivation that was refractory to three antiviral agents. No CMV-specific immunity was demonstrable before therapy, but CMV-specific CTLs became detectable 4 d after infusion, and the CMV reactivation was controlled at 11 d after infusion. Patient 8 had experienced CMV viremia for 10 wk before adoptive transfer and possessed no endogenous CMV-spe-

cific immunity. Adoptive therapy was given at day 247 after transplant and was followed by the appearance of CMV-specific CTLs in peripheral blood 7 d after transfer. Cidofovir was administered before therapy, and CMV viremia was controlled but remained detectable for several weeks before a fatal episode of bacterial sepsis. Patient 9 gained prompt immune reconstitution after adoptive transfer and long-term control of viremia.

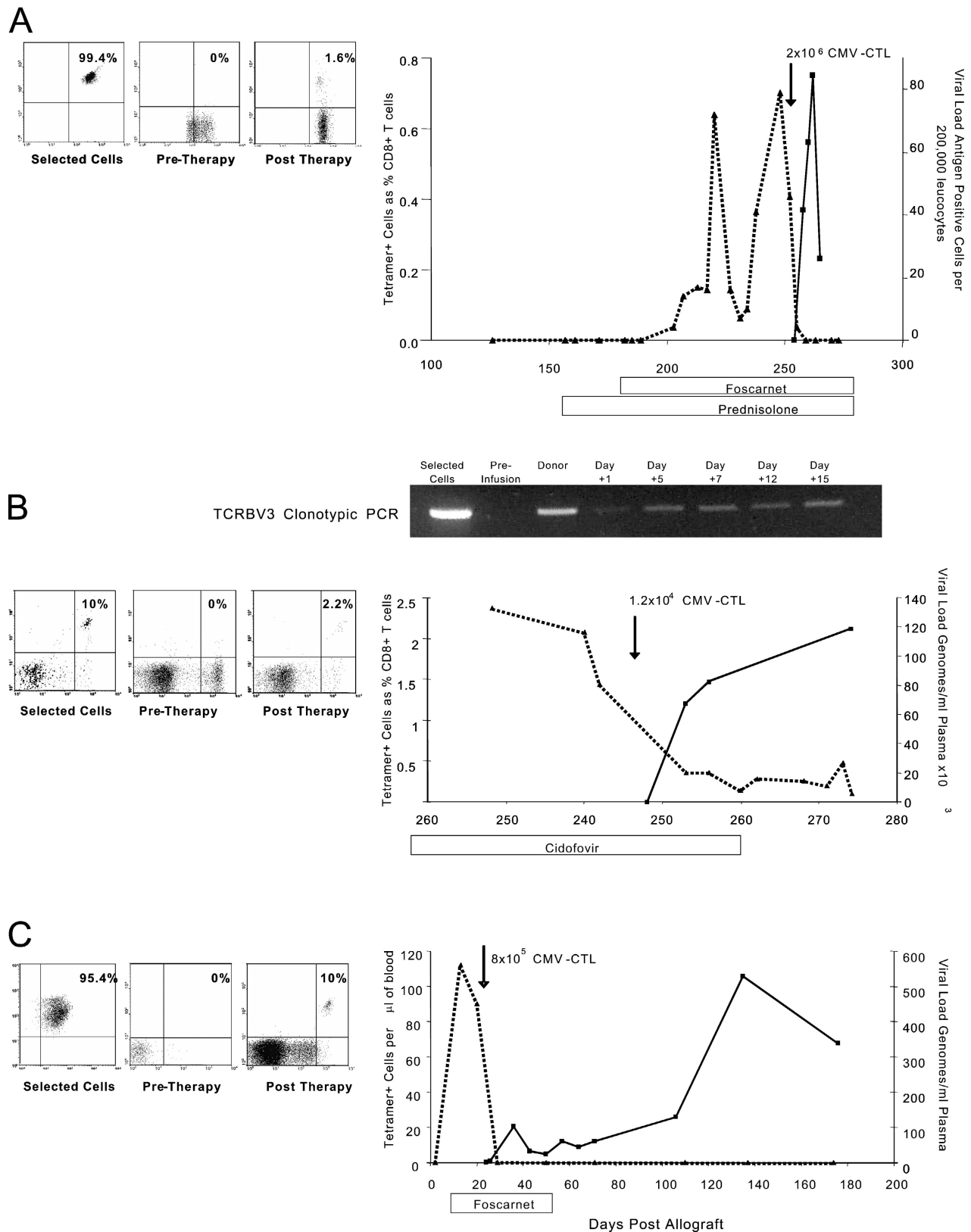


Figure 3. CMV-specific immune reconstitution and CMV viremia after adoptive transfer of CMV-specific CTLs in patients. The vertical bar indicates the timing of adoptive transfer and the absolute number of CMV-specific CTLs that was administered. Data from patients 7, 8, and 9 (A–C) is shown. ■, Absolute count of tetramer⁺ T cells or expressed as percentage of CD8 T cells; ▲, viral load. Persistence of the infused T cells was assessed in patient 7 by PCR detection of the clonotypic TCR CDR3

sequence of the infused cells in PBMC samples that were taken from the patient (Fig. 2); >90% of tetramer-binding cells expressed *TCRBV3*. A single TCR CDR3 sequence, SGEQGMDEQYFGPGTRLTVT, made up all *TCRBV3* sequences. A clonotype-specific primer (5'-gggaacagggaatggac-gag-3') was used for clonotype-specific PCR that was performed on patient PBMCs that were isolated before infusion and at days 1, 5, 7, 12, and 15 after infusion (A).

Reactivation of CMV after SCT is a consequence of the profound state of immunosuppression which is seen in patients early after transplant. This results from transplant conditioning protocols and the use of immunosuppressive drugs to suppress graft versus host disease (GvHD). It was demonstrated clearly that donor-derived CMV-specific CTLs that have been isolated and expanded in vitro may be administered to transplant patients and can control viral reactivation (2). However, only clinical units with considerable expertise can adopt such a therapy. Here, we have used HLA-peptide tetramers to transfer antigen-specific CTLs directly from the donor into patients without the requirement for in vitro culture.

CMV-specific CD8⁺ CTLs are found at high frequency in the peripheral blood of CMV-seropositive donors (6, 9), and magnetic beads can be used to select these CTLs (7, 10). We have demonstrated that this approach can be scaled up for processing of large samples of blood or leukapheresis specimens. The procedure occurs in a "closed" system using equipment that is used routinely for processing stem cell harvests; after selection, the CMV-specific CTL represented >95% of live cells.

CMV-specific T cells were administered to nine patients and no side effects were observed. Two patients exhibited mild GvHD (grades 1 and 2) within 2 wk of transfer, but grade 1 GvHD was present in both patients before infusion. The lack of significant GvHD reflects transfer of a very low number of T cells with antigenic specificity other than that for CMV. A total dose of 1.2×10^4 to 2×10^6 CMV-specific T cells was administered; this is much smaller than in other adoptive transfer protocols where up to 10^9 T cells are given. However, this number may be sufficient to control viral reactivation. Transfer of only 10^6 /kg PBMCs has been used to control posttransplant lymphoproliferative disease that is due to EBV infection (11). The frequency of EBV-specific CTLs within PBMCs is comparable to that of CMV-specific CTLs (12); this suggests that clinical efficacy was achieved by transfer of similar numbers of CTLs to that used in our study. In addition, T cells can expand in vivo by many orders of magnitude, and there was evidence for this in our study. Patient 7 had no CMV-specific CTLs before infusion; however, 6 d after transfer, these CTLs had expanded to 2×10^6 /l and represented 10^7 cells in peripheral blood. Because only 2% of the lymphocyte pool is located in the peripheral blood, this suggests a total count of $\sim 5 \times 10^8$ tetramer-binding cells—an expansion of 250-fold from the starting infusion. It is possible that T cells infused directly from donor PBMCs may be able to expand more efficiently in vivo than T cell clones which have been expanded extensively in vitro before infusion, although massive expansion of infused T cells has been reported after a period of in vitro culture (13, 14). The lymphopenic environment associated with SCT is likely to be favorable for T cell proliferation, and such a degree of expansion may not be achievable in other clinical situations.

Selected CTLs were not marked before transfer because of ethical constraints, but clinical evidence provides support

for their expansion in vivo. Endogenous CMV-specific immune reconstitution can be brisk in patients with sibling donors, but transplants from unrelated donors are associated with markedly impaired reconstitution within the first 100 d (8, 15). This is due to T cell depletion of the donor inoculum and increased histoincompatibility between donor and recipient. All three of the patients with unrelated donors in our study showed prompt immune reconstitution after adoptive therapy despite the fact that two patients had suffered from prolonged viremia before treatment.

CTLs were functional after transfer, and our findings indicate that this contributes to the control of viral replication. This is demonstrated most clearly in patient 7; his CMV reactivation was refractory to antiviral drugs but was controlled within 8 d of adoptive transfer. Prompt T cell expansion was seen in all patients, and after resolution of CMV viremia, there were no subsequent episodes of viral reactivation. The CMV-specific CD4⁺ T cell response was determined in patients 1 through 5 and showed no correlation with the degree of CMV-specific CD8⁺ T cell expansion (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20040613/DC1>).

The proportion of patients that required GCV is lower than that seen in a control group, although the degree of protection is difficult to predict given the small number of patients involved. This approach is limited to patients who express HLA alleles for which CMV peptides are available. However, many CMV epitopes have now been reported and >75% of the transplant population is eligible for treatment. This therapy is amenable to all situations in which the transplant donor has T cells that are specific for the antigen to be targeted in the patient. The most obvious clinical setting is allogeneic SCT; however, successful adoptive transfer of T cells outside the setting of SCT also was reported (16) and may be facilitated by tetramer selection. Adoptive transfer of antigen-specific CTLs after selection with HLA-peptide tetramer is a novel approach to adoptive T cell therapy that offers considerable potential for cellular therapy.

MATERIALS AND METHODS

Patients and transplant protocols. Four patients received standard myeloablative conditioning with cyclophosphamide (120 mg/kg) and total body irradiation (14.4 Gy) or busulfan (56 mg/kg/d for 4 d). Five patients received the reduced intensity conditioning regimens BEAM, CAMPATH-1H (17) or Flu/Mel/CAMPATH-1H (18). Cyclosporin and short-term methotrexate were given as prophylaxis against graft versus host disease (GvHD). Oral acyclovir was administered until day 30 after SCT.

Virus screening and antiviral therapy. Screening for CMV reactivation was performed once weekly from day 14 by qualitative PCR from whole blood and continued until day 100 (19). CMV PCR was performed on the Roche Light Cycler FastStart DNA Master (Roche Molecular Biochemicals) with quantitation over a wide range (4×10^2 to 5×10^8 copies/ml). Patient 7 was monitored using the CMV antigenemia assay, which quantifies the number of CMV infected cells in the peripheral blood per 200,000 leukocytes.

Protocol design. SCT recipients who received a transplant from a CMV-seropositive donor were recruited, and written informed consent was obtained from donor and recipient. Approval for this study was obtained from

the South Birmingham Local Research Ethics Committee. Patients who express the HLA class I alleles HLA-A*0101, HLA-A*0201, HLA-B*0702, HLA-B*0801, or HLA-B*3502 were eligible for entry. The presence of CMV-specific CTLs was determined in a blood sample from the donor; if tetramer-binding cells represented $>0.05\%$ of the CD8⁺ population they were used for large-scale selection. At the first time point of CMV reactivation, 250 ml of blood was taken from the donor, and the CMV-specific CTLs were selected. In patients 7 and 8, stored cryopreserved leukapheresis products were thawed and used to select CMV-specific CTLs. These were infused into the donor within 4 h. Standard management of CMV reactivation was not affected by the cell infusion, and GCV (10 mg/kg/d) was commenced if two positive consecutive CMV PCR tests were obtained.

Selection of CMV-specific CTLs. 250 ml of blood was mixed with an equal volume of magnetic-activated cell sorting (MACS) buffer (Miltenyi Biotec) and mononuclear cells were separated using a density gradient (Lymphoprep; Nycomed). In patients 7 and 8, the thawed leukapheresis sample was washed in MACS buffer and then treated in the same way. Cells were resuspended in 2 ml of MACS buffer and 100 μ g of 0.2 μ M filtered HLA-peptide tetramer followed by incubation at 37°C for 15 min. Cells were washed and resuspended in 2 ml of MACS buffer and 400 μ l of anti-PE paramagnetic MicroBeads (Miltenyi Biotec) at 4°C for 30 min. Tetramer-binding cells were selected magnetically using Enrichment 3.1 program on the ClinMACS^{plus} cell separator. The positive fraction was eluted in 40 ml of MACS buffer, of which 2 ml was tested for purity and cell number by flow cytometry. The remaining 38 ml was infused into the patient over 15 min. Cells were kept on ice at all times unless otherwise stated and cell manipulation was performed in a sterile cabinet in a clean room. HLA-peptide tetrameric complexes were assembled under sterile conditions, and all reagents were certified as endotoxin free. Microbiologic testing was performed on cells after magnetic selection.

Synthesis and use of tetramers. HLA tetramers were synthesized by the use of standard methods (20), and the components of the HLA-peptide tetramers that were used in the study are shown in Table I. The percentage of tetramer binding was determined by flow cytometry (EPICS XL, Beckman Coulter).

Clonotypic PCR analysis. TCR β -chain variable region gene (*TCRBV*) usage of donor CMV-specific CTLs was determined initially by FACS analysis after costaining with tetramer and TCRBV-specific antibodies (BV1-BV23, Immunotech). Tetramer-binding cells were purified ($>90\%$ purity) using magnetic selection before extraction of RNA and cDNA synthesis. *TCRBV*-specific PCR primers were used to amplify the dominant *TCRBV* family, and PCR products were cloned into a TOPO vector (Invitrogen) and sequenced (21). Clonotypic *TCRBV* PCR primers were designed complementary to the CDR3 region of the dominant transcripts. These primers were used with a *TCRBC* region primer to identify clonotypic TCR sequences within cDNA samples generated from unselected patient PBMCs that were taken at multiple time points before and after infusion of selected CTLs.

Statistical analysis. Statistical comparison was made between the proportion of patients in the study group who required treatment with GCV and those in a control group of inappropriate HLA type or donor unavailability. Because some of the expected frequencies were <5 , the chi-square test was not believed to be valid and a 95% confidence interval was determined for the difference in the proportions who required treatment.

Online supplemental material. Fig. S1 shows the functional competence of CMV-CTL. Fig. S2 shows CMV-specific CD4 T cell immune reconstitution. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20040613/DC1>.

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